INDUCTION OF IMMUNOLOGICAL TOLERANCE WITH SUBCELLULAR FRACTIONS

RICHARD J. HOWARD

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Richard J. Howard
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Introduction:

Although a modern scientific accomplishment, the transplantation of organs from an organism to one genetically different is an ancient concept. The Egyptian Sphynx is a heterograft (to use the modern terminology) between the body of a lion and the head of a human.

The idea of genetically different tissues living together in the same organism also pervaded Greek mythology. The Centaur was a heterograft between a horse and a man; the Minotaur had the body of a bull and the head and torso of a human; and the Chimera had the head of a lion, the body of a goat, and the tail of a serpent.

Modern studies on tissue transplantation began with Carrel early in this century, but it was not until 1953 that Billingham, Brent, and Medawar[1] reported the artificial induction of tolerance to tissue - in this case, skin - from one animal to a genetically different member of the same species. These investigators, using Burnet and Fenner's "self-marker" concept as a theoretical model, sttempted to destroy immunologically competent cells that could react against the tissue of strain A mice in CBA mice by injecting cells and tissue debris from testis, kidney, and spleen from A mice into fetal CBA mice. The CBA mice were challenged with skin from A mice at eight weeks of age. Whereas no skin grafts from A mice were accepted by control CBA mice that had received no injections, 60% of the treated CBA mice accepted skin grafts from A mice.

This experiment was the first successful attempt to overcome the immunological response of graft rejection. That homograft rejection is an immunological process has been known for many years. Medawar[2,3] thoroughly described the homograft rejection pattern in rabbits. Briefly, if a skin graft is transferred from a donor rabbit to an

unsensitized recipient, relatively normal healing of donor and recipient skin occurs for the first four days. When the skin is grafted, blood and lymphatic vessels from the host grow into the donor graft. Blood flow from the host to the donor skin is established, and there is little cellular infiltration for the first few days. At the end of the first week, the exact time depending on the genetic disparity of host and donor tissue, thrombosis of the vessels occurs, a leukocytic infiltrate appears, and the graft dies and is sloughed within two or three weeks.

At the time of rejection, there is an intense, leukocytic infiltration around the blood vessels in the graft and a grossly visible "black band" of cells that can be seen at the graft-host junction on the stained tissue specimen.[2] The most common infiltrating cell is the lymphocyte, but the graft also is invaded by polymorphonuclear leukocytes, immature plasma cells, and eosinophils.[4] Primary importance in the rejection phenomenon has been assigned to the production of sensitized lymph cells in the lymph nodes draining the graft site and the migration of these cells into the graft resulting in its destruction.[5,6,7]

Passive transfer experiments[8,9] have shown that regional lymph node cells are sensitized to donor antigens. If the cells from a sensitized regional lymph node are injected into the skin of the animal whose tissue was responsible for the sensitization, marked erythema and swelling may be noted at the injection site. The importance of lymphatic tissue for graft rejection is shown by prolonged survival of grafts in organs with no lymphatic drainage. Thus, the anterior chamber of the eye,[10] the brain,[10] and the testis[11,12] do not have lymphatic drainage, and homografts placed in these organs will survive for prolonged periods. Grafts placed in these organs fail to provoke an immune response. These grafts will be rejected, however, if the host is immunized against donor tissue before[13] or after[11] grafting.

Although most investigators agree that invasion of donor skin by host lymphocytes is essential for homograft rejection, the role of circulating antibodies is still debated. Medawar[13] believes classical circulating antibodies play no essential part. These antibodies may be demonstrated by a number of methods.[14,15,16] but their role in graft rejection is undetermined. Fetal lambs can reject skin grafts before they can make gamma globulin;[17] but skin grafts placed on humans with hypogammaglobulinemia survive for prolonged periods.[18] Homologous and heterologous cells placed in Millipore diffusion chambers and then inserted into the peritoneal cavity of presensitized mice were destroyed if the pores in the filter were large enough for host cells to enter but were not destroyed if only serum entered.[19,20] Another source of evidence that humoral antibodies play little role in graft rejection is the consistent failure to demonstrate passive transfer of homograft sensitivity by serum alone.[5,21] However, Haskova[22] has reported the destruction of previously accepted skin grafts in ducks by injecting serum from other ducks immunized against donor tissue.

The immunological nature of homograft rejection also is shown by the earlier and more violent rejection of a skin homograft placed on a previously sensitized host.[2,4] The "second set reaction" is characterized by earlier rejection beginning within the first week of grafting. In this type of rejection, graft tissue is not invaded by host blood vessels, and the graft never becomes vascularized. No time is needed for sensitization of the host, and the graft is quickly and violently rejected, Failure of vascularization causes the graft to turn white for lack of blood - hence, the alternate name - "white graft reaction."

The preceding paragraphs attempt to show that the phenomenon of

graft rejection involves an immunological process. Investigators trying to induce tolerance to foreign grafts have sought methods of surpressing the immune response long enough for the donor tissue to become part of the host or to abolish completely the host's reaction against the donor.

The first successful induction of tolerance in 1953 by Billingham, Brent, and Medawar[1] was, in part, a test of the immunological theory of Burnet and Fenner.[23,24] In turn, Burnet and Fenner based their "self marker concept" partly on the work of Owen.[25,26] In 1945, Owen discovered that dizygotic twin cattle had, in addition to their own red blood cells, the same type of erythrocytes as their twin. After Lillie[27] found that dizygotic cattle often share a common blood supply in utero, Owen proposed that erythrocytes and erythropoeitic cells were freely exchanged during the embryonic period and were able to survive in the mature animal. This red cell chimerism has also been demonstrated in sheep,[28] chickens,[29] and man.[30,31]

Burnet and Fenner's theory of immunologic specificity proposed that during the embryonic life the organism learns to recognize its own antigens as "self" so that it is unable to react against them later. On the other hand, if the animal is exposed to antigens after birth, it recognizes them as "not self" and produces antibodies against them.

On the basis of this theory and Owen's discovery of red cell chimerism is cattle, Burnet and Fenner predicted that foreign material introduced into the embryo would be recognized as "self", and the animal would be unable to react immunologically against them later. Burnet et al.[32] failed to provide experimental evidence for this prediction, using antibody production in the chicken as the experimental model.

Other investigators, however, were able to provide evidence for Burnet and Fenner's theory. In 1950, Gross[33] discovered that lymphatic

leukemia would cause leukemia in another inbred mouse strain if the cells were transplanted to newborn mice but not if they were transplanted to older mice of that strain. But Kaliss and Snell[34] made successful homotransplantations of mouse tumors after giving injections of lyophilized kidney, liver, and spleen from the donor strain.

Anderson et al.,[35] in 1951, and Billingham et al.,[36] one year later, added to Owen's findings and supplied evidence for Burnet and Fenner's prediction, when the skin grafts they exchanged between dizygotic twin cattle survived permanently.

It was not until 1953 that Billingham, Brent, and Medawar[1] first artificially produced immunological tolerance to skin homografts in mice. The authors also describe the induction of acquired tolerance to skin grafts in chickens by injecting donor blood into the chick embryo. Skin transplanted to the prepared host after hatching survived permanently.

Although this paper deals with skin transplantation in mice, many other species are being used to study transplantation phenomena: hagfish, paddlefish,[37] lamprey,[37] chickens,[1,38,39,53] ducks,[22] hamsters, rats,[40,41] guinea pigs,[42] cattle,[35,36,44,46,50] rabbits,[43] turkeys,[38,39] and pheasants.[38]

Tissues other than skin have been used to investigate transplantation: tumor,[34,45] kidney,[46] liver,[47] endocrine gland,[11,48] spleen,[49] lung,[50] and heart.[51]

Billingham et al.[52] believed donor cells had to be injected before birth to establish tolerance to future skin grafts. Optimal time for injecting foreign cells depended on the species, since some aminals develop immunological competence during the embryonic period. The few days before and after birth in the mouse were a "null period" during which foreign cells elicited neither tolerance nor immunity. For each species there was an optimal period for the induction of immunological tolerance.

However, in 1955, Woodruff and Simpson[40] and Egdahl et al.[43] reported that tolerance to skin homografts could be induced in the rat by injecting spleen cells as late as two weeks after birth. Other workers also demonstrated that immunological tolerance could be produced after birth. As far back as 1951 Kaliss and Snell[34] reported growth of transplanted mouse tumor following injection of lyophilized donor spleen, kidney, and liver after birth. Others showed tolerance also could be produced in chickens after hatching.

Similarly, Aust et al.[54] produced tolerance to an adenocarcinoma in A strain mice by injecting tumor cells of the C3H strain a few hours after the host was born. However, tolerance could not be produced if the tumor cells were transplanted in the adult animal.

In 1957, Billingham and Brent[38,55,56] and Billingham[57] and Martinez et al.[58] provided evidence that acquired immunological tolerance could be produced in the newborn mouse by intravenous or intraperitoneal injection of spleen cells from the donor mouse strain. Tolerance could not be produced if the cells were injected into the subcutaneous tissues of the host.

Gombos[46] has reported successful homotransplantation of kidneys in young dogs by completely replacing their blood with blood from the future donor. However, Fowler[44] showed no prolongation of survival of skin homografts in puppies following blood and leukocyte transfusions.

When, in 1959, Shapiro et al.[59] and Mariani et al.[60] showed immunological tolerance could be produced in adult animals as well, Burnet and Fenner's explanation of immunological tolerance had to be revised. Thus Mariani et al.[60] were able to induce tolerance to skin isografts across the male-female histocompatability barrier in C57Bl/1 and A strain mice by injecting the adult female mice (who normally reject

skin from male mice of the same strain because of a sex-linked histocompatability barrier) with twenty million spleen cells or by making the male and female mice parabiotic partners. These experiments and others[61-69] showing tolerance could be produced in adult mice called for revising Burnet and Fenner's "self marker concept".

The similarity of immunological tolerance and the inhibition of the immune system by antigen overloading (immunological paralysis) has been pointed out by Good et al.[60,70,71,72] Immunological paralysis is the inhibition of immunization and sensitization to antigens caused by injecting large quantities of antigen. Through such intramuscular injections, Sulzberger[73,74] demonstrated the prevention of sensitization to neoarsphenamine in guinea pigs. Immunological paralysis to pneumococcal polysaccharide was demonstrated by Felton and Ottinger[75] and by Felton.[76] They showed that whereas 0.0005 mg. of pneumococcal polysaccharide would immunize the mouse against the pheumococcus, 0.5 mg. of pneumococcal polysaccharide would not protect the mouse from the pneumococcus.

Immunological tolerance or "immunological unresponsiveness" to protein antigens by the injection of large doses of the antigen has been demonstrated in rabbits by a number of investigators.[77-80] This unresponsiveness was specific for the protein administered, the antibody production to other proteins being unaffected.

Tolerance to cellular antigens was demonstrated by Mitchison.[81] He was able to maintain homologous erythrocytes labeled with Cr^{51} in the circulation of chickens by repeated administration of red blood cells. The transferred red cells were eliminated by a nonimmune mechanism.

In all these experiments, the necessity of the persistence of antigen for the maintainance of immunological unresponsiveness is evident.

Other mechanisms of immunological tolerance have been reviewed by Hasek et al.[82] These investigators have proposed that antigen

responsible for the induction of tolerance could activate an adaptive enzyme so that the antigen would be broken down by a mechanism other than that which leads to immunity. Another mechanism is that the tissue antigen has an instructive function causing some change in the genetic structure of the mesenchymal cells or transfer of its own genetic material to the host that would inhibit the formation of antibodies against grafted material. Experimental evidence is lacking for these theories.

Most investigators believe antigen must be present for the maintainance of tolerance.[73,81,82] Good et al.[70-72,83] have drawn a direct parallel between immunological tolerance and immunological unresponsiveness. Both these phenomena are similar in specificity, necessary persistence of antigen, greater ease of induction in very young animals, and possibility of producing unresponsiveness in adult animals with large quantities of antigen.

The argument has been that lymphoid cells given to the fetus or neonate have survived and multiplied in the host while constantly putting out tissue antigen. This constant supply of antigen to the host has created a state similar to immunological paralysis produced by Felton with pneumococcal polysaccharide. When skin subsequently was grafted, the host was unable to react against the graft because the antigens in the graft that normally immunize the host were the same as those produced by the lymphoid cells with which it was previously injected and to whose antigens it is now unable to respond. It has been demonstrated[84] that mice made tolerant are chimeras in their lymphoid cell populations; they have both host and donor cells. Since these cells are living in the host, they always are producing isoantigens, thus keeping the host animal loaded with donor antigen and consequently unresponsive and tolerant. The skin homograft continues to produce antigen as well and adds to the tolerance-producing antigen production of the previously

injected donor cells.

This explanation predicts that immunological tolerance is a dose-response relationship. Small doses of antigen produce immunity; large doses of the same antigen cause immunological unresponsiveness or tolerance. Thus, even very young animal might be immunized if given small enough doses of antigen. Howard et al.[85,86] have shown that small quantities of homologous spleen cells given to newborn mice could produce immunity. Fetal lambs,[17] guinea pigs,[87] and human neonates[88] also are capable of immunological responses.

The theory paralleling immunological tolerance to immunological paralysis also suggests tolerance can be produced in any animal of any age with enough antigen. Antigen must be given in large doses repeatedly, since it is constantly being metabolized by the host. In 1961, Shapiro et al.[64] induced tolerance to (A x C3H) F_1 hybrid skin grafts in adult C3H mice by administering 1,500 million donor cells to the host C3H mice in seven to eleven weeks.

Tolerance also has been induced in mice with cell-free extracts of lymphoid cells. In 1960, Billingham and Silvers[89] were able to induce tolerance to skin of C57 male mice in C57 female mice by the previous administration of a cell-free extract of male lymphoid cells. Linder[90] induced tolerance across the male-female histocompatability barrier in adult mice with homogenates of kideny, spleen, and liver. Martinez et al.[72,91] and Kelly and Brown[92] also induced tolerance across the male-female barrier in C57Bl/1 mice, using cell-free preparations of liver, kidney, heart, and blood. And Martinez et al.[70] produced tolerance to homologous skin grafts in mice across stronger histocompatability barriers including the strong H-2 locus with disrupted spleen cells.

The following experiments are an extension of the work previously mentioned which show the capability of disrupted cells and subcellular

fractions to induce immunological tolerance. They are an attempt to define further the cell constituents responsible for tolerance. Part of this work has been published.[71]

Methods:

Animals: Mice of the C57Bl/1, A, C3H, and BALB/C strains were used in these experiments. Eichwald and Silmser[97] first reported the inability of C57Bl/1 female mice to accept skin isografts from C57Bl/1 male mice, but female skin grafts were uniformly accepted by male mice. This finding is thought[98,99] to be due to the presence of a histocompatability gene on the Y chromosome. Since this combination is a weak histocompatability barrier, it provides a sensitive method for testing the induction of immunological tolerance. The mice were obtained originally from the colony of Dr. J. J. Bittner and have been inbred for over 100 generations.

In these experiments, female C57Bl/1 mice were from 45 to 75 days old when the experiment was begun. Spleens, livers, and skin grafts were taken from male mice ranging in age from 45 to 180 days old.

In other parts of these experiments, mice of the A, C3H, and BALB/C strains were used. These mice also were obtained from the colony of Dr. Bittner and have been inbred for over 100 generations. The BALB/C mice were 90 to 180 days old when skin grafts were removed.

Newborn C3H mice received injections of spleen fractions and were 35 days old at the time of grafting. The A strain mice ranged from 60 to 360 days old.

The C57Bl/1 and BALB/C mice differ at the H-2 histocompatability barrier; the A and C3H mice also differ at this barrier.[100]

Preparation of Antigenic Material: Male C57Bl/1 mice were sacrificed with ether anesthesia, their skins were cleaned with 70% alcohol, and their spleens were immediately excised and placed in ice-cold lactate-Ringer's saline solution (Cutter). The spleens were reduced to a paste in a tightly fitted, ground glass homogenizer with an internal diameter

of one-half inch containing two to four milliliters of lactate-Ringer's saline solution. Cells were disrupted by subjecting the paste to four cycles of freezing and thawing. Solid carbondioxide (-60° C.) was used for freezing, and warm tap water was used for thawing. Microscopic examination confirmed that no intact cells remained after the last cycle of freezing and thawing. The spleen capsules were removed by filtering the homogenate through surgical gauze.

The spleen cell homogenate was separated into subcellular fractions by centrifugation. The particulate fraction was the precipitate removed by centrifugation of the spleen cell homogenate at 15,000 g for 10 minutes in a Spinco Model L ultracentrifuge with a number 40 rotor. This precipitate contained cell walls, nuclei, mitochondria, and other large cell debris.

The supernate then was centrifuged at 105,000 g for 60 minutes. The sediment from this centrifugation, containing the cell microsomes was termed the microsomal fraction. The soluble fraction was the remaining supernate. Each fraction was washed and suspended in lactate-Ringer's solution. The concentration was two spleen equivalents per milliliter. (A spleen equivalent is that amount of material removed from one spleen.) These fractions were prepared from the spleens of A strain mice in an identical manner.

In addition, a "Tris"-extracted particulate fraction and a RNAse-treated microsomal fraction were prepared from the spleens of C57Bl/1 male mice.

The "Tris"-extracted particulate fraction was prepared by extracting the particulate fraction with 0.1 M trihydroxy-methyl-amino-ethane ("Tris") pH 8.7 for 30 minutes at 25° C. After centrifugation at 15,000 g for 20 minutes, the precipitate was suspended in lactate-Ringer's

saline solution with a final concentration of two spleen equivalents per milliliter.

Preparation of the RNAse-treated microsomal fraction included the suspension of the microsomal fraction in normal saline with a concentration of four spleen equivalents per milliliter and incubation with 0.05 mg. bovine pancreatic RNAse (Sigma Chemical Company) in 0.01M "Tris" acetate buffer, pH 7.5; per milliliter microsome suspension. This mixture was dialysed at 4° C. against 0.01 M "Tris" buffer for 24 hours. After this period, a test for RNA was made by the orcinol method on an aliquot of the sample. This test was uniformly negative.

In a subsequent experiment, the microsomal fraction was divided into a ribosomal fraction and a microsomal membrane fraction. Ribosomes were separated from the microsomes of the spleens of C57Bl/1 male mice according to Korner's method.[101] The spleen cells were disrupted in a ground glass homogenizer. After the large cell particles were removed by centrifugation at 15,000 g for 10 minutes, the supernate was collected and one-ninth its volume of five per cent sodium deoxycholate in 0.03 M "Tris" buffer, pH 8.2 was added. The ribosomes could be isolated from the membranes by centrifugation at 105,000 mg for two hours. To the ribosomes isolated from 25 spleens (approximately 1.75 gm. wet weight) was added 0.02 mg. bovine pancreatic RNAse (Sigma Chemical Company) in 0.001 M "Tris" acetate buffer and 0.005 M ethylene-diamine-tetraacetic acid (EDTA), pH 7.5. This mixture was incubated at room temperature (25° C.) for one hour and then kept at 4° C. for 17 hours. These two fractions were then suspended in normal slaine, one-half spleen equivalen per milliliter, for injection.

In another experiment, liver tissue of C57Bl/1 male mice was used to prepare a microsomal fraction and a particulate fraction. Livers of C57Bl/1 male mice were excised and frozen at -20° C. until

used (one day to two weeks). A total of 231 gm. of liver (wet weight) was used in this experiment. The frozen liver tissue was thawed and homogenized in a Waring blender and a ground glass homogenizer. Large pieces of particulate matter were removed by centrifugation at 15,000 g for 10 minutes in a Spinco Model L-2 ultracentrifuge in a number 30 rotor. The supernate was then centrifuged at 105,000 g for two hours.

The pellet from this centrifugation was dispersed in five per cent sodium deoxycholate in isotonic sodium chloride solution. The mixture was then centrifuged for two hours at 105,000 g in a Spinco zonal ultracentrifuge using a 30% to 50% sucrose gradient The contents of the rotor were withdrawn in forty 40 ml. aliquots as the rotor was spinning at 4,000 g. Relative protein concentraction of each aliquot was estimated by recording the optical density of the sample at a wavelength of 280 mu on a Beckman Model D spectrophotometer. Five peaks were found and were determined to correspond to Svedberg units of 10S, 30S, 50S, and 120S. The fifth peak represented the material at the bottom of the rotor. The aliquots corresponding to the 120S peak (determined to be the liver microsomes) were dialysed against 0.001 M "Tris" buffer, pH 7.4, for four days at 4^{o} C., at the end of which the white precipitate was collected by centrifugation at 105,000 g for 60 minutes.

The aliquots representing the fifth peak, the particulate matter, were centrifuged at 105,000 g for two hours. The pellets from these two fractions were divided into 13 portions and stored at -20^{o} C. until used.

Injections: Fractions prepared from C57Bl/1 male spleens were injected into C57Bl/1 female mice twice weekly. The first injection was given intravenously through a tail vein. Fifty USP units of heparin (Liquaemin Sodium, Organon, Inc.) was given before the intravenous injections.

Each injection included one spleen equivalent of that fraction. Subsequent injections were performed intraperitoneally. Some mice died after the intravenous injection due to embolization of the injected material. These animals were excluded from the results. Skin grafting was performed after the fourth injection, and semiweekly injections were continued for an additional four weeks.

The particulate and microsomal fractions obtained from C57Bl/1 male livers were injected into female mice in 13 equal parts. Intravenous administration was alternated with intraperitoneal injection. No heparin was given before the intravenous injections. The C57Bl/1 female mice were given seven injections in a period of ten days and then were grafted with skin from C57Bl/1 male mice. Six injections were given in the ten days after grafting.

The injection schedule of spleen fractions from A strain mice into newborn C3H mice was 0.2 spleen equivalent three times per week for the first week, 0.5 spleen equivalent three times per week for the next two weeks, and one spleen equivalent three times per week for the next two weeks. Tumor grafts were placed subcutaneously into the C3H mice at the end of the fifth week. Control mice that received no injections also were grafted at five weeks of age. One spleen equivalen was injected twice weekly thereafter into the experimental animals until graft acceptance or rejection was determined (approximately four weeks). All injections were given intraperitoneally.

Control C57Bl/1 female mice were injected with "Tris" buffer solution or the RNAse solution and followed the same injection as the experimental mice. Other control mice received no injections.

Skin Grafts: Skin grafts from C57Bl/1 male to C57Bl/1 female mice were administered by the standard method of this laboratory.[58] After the mice were anesthetized with sodium nembutal, the hair was clipped

from the ventral aspect of the male donors and the dorsum of the female recipients. The skin was washed with 70% alcohol, and a two centimeter square piece of skin was removed from the ventral surface of the male donor, rotated 180°, and placed on a graft bed of the same size on the back of the female host. The graft bed had been prepared previously by removing a similar sized piece of skin from the female. The skin grafts were secured in place with interrupted 5-0 silk sutures or with metal skin clips. Reversing the grafts facilitated the evaluation of subsequent graft acceptance or rejection, since the hair of the graft grows in a direction opposite to the host's.

The grafts were evaluated by gross inspection. The criteria for graft acceptance (and thus for immunological tolerance) were lack of inflammatory or indurative signs (i. e. rejection) and growth of new hair 60 days after grafting. Skin grafts were observed for nine months.

To determine whether tolerance produced by the spleen or liver fractions was specific for that tissue genotype or was merely a nonspecific inhibition of the immune response, some of the animals tolerant to C57Bl/1 male skin grafts were grafted with skin from BALB/C mice. The tolerant C57Bl/1 mice were grafted after the male skin had been in place six months,

Tumor Grafts: A mammary adenocarcinoma from A strain mice was grafted to C3H mice. A small peice of tumor, approximately two millimeters on a side, was placed subcutaneously into the left groin of C3H mice through a small incision in the left flank. Ether anesthesia was used, and the incision was closed with a singly 5-0 silk suture or skin clip. The criterion for acceptance of the tumor was growth of a mass at the site of implantation with the eventual death of the host. These masses grew to be several centimeters long by the time the mouse died. In nontolerant mice, no mass could be detected.

Results:

The results of the skin grafts placed on C57Bl/l female mice receiving subcellular fractions from male C57Bl/l mice are shown in Table I. The microsomal fraction and particulate fraction possess the ability to induce immunological tolerance to male skin isografts in female mice. Five of seven (71%) female mice injected with the particulate fraction accepted male skin grafts, and six of eight (75%) female mice receiving the microsomal fraction accepted male skin grafts. Only two of eight (25%) female mice that were given the soluble fraction accepted syngeneic male skin grafts. Of the uninjected control mice, only two of fifty-nine (3%) accepted male skin grafts.

Figure I shows the female control mice which had been grafted with male skin. The grafts were applied 70 days before the photograph was taken. Only shrunken scar tissue remains where the graft was. Figure II shows 70 day-old male skin grafts on the dorsum of female mice injected with the microsomal fraction. The grafts did not shrink and show a luxuriant growth of hair. All the host's hair remained black, but some of the donor hair lost its pigmentation following grafting. Graft hair was coarser and lacked the sheen of the host hair.

Table I also indicates that destruction of the microsomal RNA by bovine pancreatic RNAse did not appreciably alter the ability of this fraction to induce tolerance. Six of eleven (55%) female mice had intact male skin grafts at the end of two months. The grafts were still viable when the animals were sacrificed nine months after grafting. Three of the mice can be seen in Figure III, which shows the coarse, white hair of the grafts. The hair of the host mice was not completely regrown at the time this photograph was taken, approximately 40 days after grafting.

Extraction of the particulate fraction with "Tris" buffer did not weaken the ability of this fraction to produce tolerance. Nine of eleven (81%) mice retained their skin grafts for at least nine months. The control mice injected with either "Tris" buffer or the RNAse solution showed no graft acceptances, none of twelve and none of ten, respectively.

Also included in Table I are the survival data of male skin grafts on C57Bl/1 female mice that received the ribosomal fraction and the microsomal membrane fraction. Of twenty female mice injected with ribosomes prepared from the spleens of C57Bl/1 male mice, four (20%) retained skin grafts with good hair growth longer than two months. Two of eight (25%) injected with the microsomal membrane fraction had intact skin grafts at the end of two months. The grafts in both groups remained in place with no sign of rejection for an additional six months.

Table II shows the results of grafts acceptance in the C57Bl/1 female mice injected with subcellular fractions prepared from the livers of C57Bl/1 male mice. Nineteen of twenty-two (86%) female mice injected with liver particulate fraction had healthy, intact grafts at the end of eight months. Sixteen female mice were injected with the liver microsomal fraction, and twelve (75%) of them retained the male skin grafts for at least eight months.

Thirty-four C57Bl/1 female mice tolerant to male skin grafts were grafted with BALB/C skin when the C57Bl/1 male skin grafts had been in place for six months. None of these grafts survived more than one week, the same as control mice. Their rejection did not affect the survival of the intact male skin grafts. These data can be seen in Table III.

Table IV shows the results of the attempt to induce tolerance across the H-2 histocompatability barrier in C3H mice to mammary adenocarcinoma of Astrain mice. Six of seven (86%) C3H mice

injected with the microsomal fraction accepted A tumor grafts; the tumors eventually caused their death. Similarly, five of eight (63%) C3H mice treated with the sedimented particulate fraction and two of seven (28%) C3H mice receiving the soluble fraction accepted tumor grafts from A strain mice. None of the ten control mice that received no injections showed evidence of tumor growth.

Discussion:

It has been little more than a decade since Billingham, Brent, and Medawar[1] first artificially induced immunological tolerance in fetal mice by injecting them with viable donor spleen cells. Since that time practical accomplishment of transplantation and theoretical speculation have made much progress. From discoveries made in the mouse and dog, man has come to benefit from progress in kidney transplantation. Lung, liver, and heart transplantation also have been performed in humans.

The present experiments and others have necessitated a reconsideration of the theories of immunological tolerance. Using Burnet and Fenner's[23,24] "self marker concept" and clonal selection theory, early investigators explained that induction of tolerance in mice had to be effected during the intrauterine period or within one or two days of birth. The clone of cells directed against the injected donor cell antigens could be fooled into believing these antigens were actually part of itself (the host); the clone of cells that would be directed against the donor cell antigens if injected at a later date, would conveniently die or be surpressed so that the animal could not react against the antigens in a subsequent graft from the original donor animal (or a genetically identical one).

It was not long, however, before this theory had to be revised and then discarded entirely. Other workers[40,43,59,60] showed that immunological tolerance could be accomplished after the immediated neonatal pariod and even during adult life when the host was immunologically mature. (It also has been demonstrated that fetal and newborn animals have the ability to react against antigenic stimulation[17,85-88]). It appears that the age at which an animal is made tolerant is not as

important as the genetic disparity between the donor and the host animal. The number of cells or amount of antigenic material needed for tolerance seem to be directly proportional to the genetic differences between the donor and host. As stated before, the group from Minnesota under Good has drawn a direct parallel between the phenomenon of immunological tolerance and the inhibition of the immune response by antigen overloading. To prevent production of antibodies and, hence, immunity, large quantities or repeated injections of polysaccharide or protein antigens had to be given. As long as these antigens were present in the host, no antibody production could be demonstrated.

The situation is analogous in producing immunological tolerance. A relatively small number of donor cells can produce tolerance in fetal or new born animals which have relatively few cells capable of producing antibodies (cellular or humoral) to the specific antigens of the foreign cells. By surviving and proliferating in the host the donor cells continuously produce antigens and keep the host in an unresponsive or paralyzed state. The adult, because of higher numbers of antibody-producing cells, requires more donor antigen to reach the unresponsive state. Therefore, more cells must be administered to an animal that is older to render it unresponsive.

The present as well as previous studies[70,72,89-92] show that live cells are not necessary for inducing immunological tolerance. By repeated administrationg of disrupted cells from the spleen, liver, or kidney or of subcellular fractions from spleen or liver cells, it is possible to prevent the host from reacting against subsequent skin or tumor grafts. The fate of this material, once injected into the host, has not been examined, but it may be proposed that examination of the

reticuloendothelial cells would provide evidence of antigenic material from the donor animal. Grafts remaining on the host animal until its death show that tolerance produced by subcellular fractions is permanent.

The experiments described here offer a suggestion of what part of of the cell contains the factor or factors responsible for the induction of immunological tolerance. On the basis of these experiments, it appears that the crude particulate fraction made up of cell wall, nuclei, mitochondria,and other cell debris, and the microsomal fraction both contain material capable of inducing a high degree of immunological tolerance in C57Bl/1 female mice to C57Bl/1 male skin isografts. Seventy-one per cent (5/7*) of the female mice injected with the particulate fraction accepted syngeneic male skin grafts, and seventy-five per cent (6/8) of the female mice receiving microsomal fraction were made tolerant to skin grafts from male mice. Little tolerance-producing ability is present in the soluble fraction; only twenty-five per cent (2/8) of the female mice accepted syngeneic male skin grafts.

Extraction of the particulate fraction with "Tris" buffer did not diminish its ability to induce immunological tolerance; indeed, eighty-one per cent (9/11) of the female mice injected with this fraction and later grafted with male skin grafts kept the grafts intact at the end of nine months. Similarly, destruction of the RNA did not lower appreciably the ability of the RNAse-treated microsomal fraction to effect tolerance to male skin grafts in syngeneic female mice. Fifty-five per cent (6/11) of the C57Bl/1 female mice injected with

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* number of grafts accepted/ number of animal grafted

the RNAse-treated microsomal fraction were made tolerant to C57Bl/1 male skin grafts.

However, when ribosomes were separated from the microsomal membranes and these two fractions administered separately to female mice, male skin graft acceptance was reduced markedly. Only twenty per cent (4/20) of the female mice injected with spleen ribosomes had viable male skin grafts at the end of two months. Twenty-five per cent (2/8) of the female mice receiving microsomal membrane fraction accepted male skin grafts.

Table II shows that liver fractions also can induce a high degree of immunological tolerance in C57Bl/1 female mice to skin grafts from male C57Bl/1 mice. Of those mice receiving the particulate fraction 86 per cent (19/22) of the mice retained male skin isografts longer than two months. At the present time, the grafts have been in place for eight months and show no sign of rejection. Seventy-five per cent (12/16) of the female mice receiving liver microsomal fraction had intact male skin grafts longer than two months. These grafts also show no sign of rejection at the end of eight months.

It can be argued that the effect of injecting these various spleen and liver fractions does not produce specific immunological tolerance. Rather, they may act as general inhibitors of the immunological response in a manner similar to substances that blokade the reticulo-endothelial system. If this hypothesis were true, the C57Bl/1 female mice tolerant of male skin grafts would not be able to reject skin grafts from other strains. However, these tolerant C57Bl/1 female mice did reject skin grafts from BALB/C mice in the normal period of one week. This finding provides evidence that the tolerance produced by the subcellular fractions is specific. It can be argued, however,

that the antigenic challenge presented by the two grafts was not comparable, because the male-female histocompatability difference is very small, whereas C57Bl/l and BALB/C mice differ at the H-2 locus, a formidable histocompatability barrier.

The results of the tumor grafts from A to C3H mice demonstrate that immunological tolerance also can be effected across the H-2 histocompatability barrier by the administration of subcellular spleen fractions of the donor strain. A high percentage of tumor grafts were accepted in the C3H mouse treated with the microsomal fraction, .86% (6/7), with the particulate fraction, 63% (5/8). A much lower incidence of acceptance was accomplished with the soluble fraction; 28% (2/7) of the C3H mice accepted tumor grafts from A strain mice.

Because of the impurity of these fractions, it is impossible to disclose the exact chemical nature of the material that induces immunological tolerance. It is possible that the particulate fraction was contaminated by microsomes, but it seems less possible that the microsomes were contaminated by larger cell debris. That multiple factors are capable of inducing tolerance is likely, and previous work has shown that more than one chemical species can function as transplantation antigens.

The results of the experiments reported here differ from those of previous studies[93-95] which have shown prolonged survival of homografts in the rat by the administration of donor RNA. Indeed, Traktellis et al.[96] were able to produce permanent tolerance in C57Bl/6 female mice to C57Bl/6 male skin grafts with spleen ribosomes and RNA extracted from male spleens. In a preliminary study, we also were able to duplicate these results. Tolerance to male C57Bl/l skin grafts was induced in newborn female mice by the administration of

150-200 ug. RNA extracted from male spleen. Skin grafts were applied at one month of age, and 89% (8/9) of the female mice grafted have retained male skin grafts for longer than eight months.

In these experiments, destruction of the microsomal RNA did no reduce appreciably the ability of that fraction to induce immunological tolerance. And yet the administration of RNA also was able to produce tolerance. One may argue that both the RNAse-treated microsomes and the RNA act as antigens. Their excess supressed the immune response by antigen overloading. This argument would be consistent witn the apparent finding that there is not one "transplantation antigen" but a variety of them. It seems unlikely, however, that the small quantity of RNA, 200 ug., would provide sufficient antigen to paralyze the immune response.

A more reasonable hypothesis is that the RNA injected into newborn mice enters their cells and provides information for the formation of protein antigens that can paralyze the immune response. Since donor RNA is used, the proteins produced by the host would have the structure of donor protein. The finding by Traktellis et al.[96] that microsomal but not soluble RNA can induce tolerance favors this explanation.

However, no one has shown what happens to the RNA inside the host animal. The high turnover of RNA may preclude its presence in the host animal long enough for the production of sufficient protein to supress the immune response.

The findings of these experiments provide further evidence that tolerance is not a unique phenomenon, but is essentially the same as immunological paralysis and inhibition of the immune response by antigen overloading.

Table I. Acceptance of C57Bl/1 Male Skin Grafts by Adult C57Bl/1 Female Mice after Multiple Injections of Subcellular Spleen Fractions.

Cell Fraction	No. of Injections*	No. takes/ No. grafted	% Graft Acceptances
Uninjected control	0	2/59	3
RNAse control	11	0/10	0
"Tris" buffer control	11	0/12	0
Particulate fraction	12	5/7	71
Microsomal fraction	12	6/8	75
Soluble fraction	12	2/8	25
RNAse-treated microsomal fraction	11	6/11	55
"Tris"-extracted particulate fraction	11	9/11	81
Ribosomal fraction	8	4/20	20
Microsomal membrane fraction	8	2/8	25

*one spleen-equivalent per injection. (avg. wt. = 0.07 gm. / spleen.)

Table II. Acceptance of C57Bl/1 Male Skin Grafts by Adult C57Bl/1 Female Mice after Multiple Injections of Subcellular Liver Fractions.

Cell Fraction	No. of Injections*	No. takes/ No. grafted	% Graft Acceptances
Uninjected control	0	2/59	3
Particulate fraction	13	19/22	86
Microsomal fraction	13	12/16	75

*each injection represents the amount of that fraction derived from approximately 0.8 gm. liver (wet weight).

Table III. Acceptance of BALB/C Skin Grafts by Adult C57Bl/1 Female Mice with Intact Skin Grafts from C57Bl/1 Male Mice.

Fraction with which C57Bl/1 female mice were made tolerant to C57Bl/1 male skin grafts	No. takes/ No. grafted	% Graft Acceptances
Spleen ribosomal fraction	0/3	0
Spleen microsomal membrance fraction	0/2	0
Liver particulate fraction	0/18	0
Liver microsomal fraction	0/11	0
Total	0/34	0

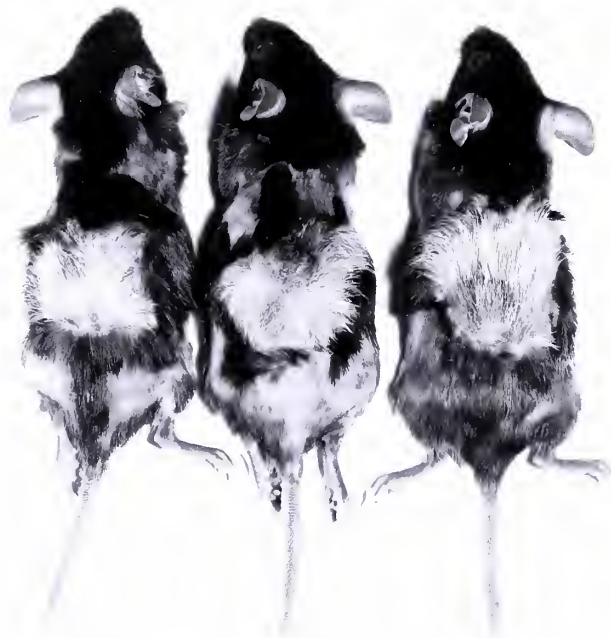
Table IV. Acceptance of Mammary Adenocarcinoma Grafts of Strain A Mice by C3H Mice after Multiple Injections of Subcellular Spleen Fractions.

Cell Fraction	No. Spleen Equivalents	No. takes/ No. grafted	% Graft Acceptances
Uninjected control	0	0/10	0
Soluble fraction	24	2/7	28
Microsomal fraction	24	6/7	86
Particulate fraction	24	5/8	63

Figure I. Photograph of control mice taken 70 days after grafting with male skin. The skin grafts are shrunken and are represented only by a small piece of scar tissue. There is no hair growth.

Figure II. Photograph of C57Bl/1 female mice made tolerant to male skin grafts with the spleen microsome fraction. The grafts maintain their original size, and a luxuriant growth of hair can be seen.

Figure III. Photograph of C57Bl/1 female mice made tolerant to C57Bl/1 male skin by previous treatment with the RNAse-treated microsomal fraction. Although all of the host's hair has not yet regrown, a good growth of hair on the grafts can be seen. The hair on the graft is longer and coarser than the host's hair and has lost its pigmentation. This picture was taken forty days after grafting.



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